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GEK1, a gene product of *Arabidopsis thaliana* involved in ethanol tolerance, is a D-aminoacyl-tRNA deacylase

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ABSTRACT

GEK1, an *Arabidopsis thaliana* gene product, was recently identified through its involvement in ethanol tolerance. Later, this protein was shown to display 26% strict identity with archaeal D-Tyr-tRNA^{Tyr} deacylases. To determine whether it actually possessed deacylase activity, the product of the *GEK1* open reading frame was expressed in *Escherichia coli* from a multi-copy plasmid. Purified *GEK1* protein contains two zinc ions and proves to be a broad-specific, markedly active D-aminoacyl-tRNA deacylase *in vitro*. Moreover, *GEK1* expression is capable of functionally compensating in *E. coli* for the absence of endogenous D-Tyr-tRNA^{Tyr} deacylase. Possible connections between exposure of plants to ethanol/acetaldehyde and misaminoacylation of tRNA by D-amino acids are considered.

INTRODUCTION

Several aminoacyl-tRNA synthetases have been reported to have the ability to transfer the D-isomer of their amino acid onto their cognate tRNA. Depending on the synthetase, the rates of such misacylations are 15–2000-fold lower than the rates obtained with the cognate L-amino acids (1,2). However, these rates are high enough to cause accumulation in the cell of metabolically inactive D-aminoacyl-tRNAs (3). To recycle such misacylated tRNA molecules, cells display enzyme activities capable of hydrolyzing the ester bond between the polynucleotide and the D-amino acid. These activities were detected for the first time in *Escherichia coli*, yeast, rabbit reticulocytes and rat liver by Calendar and Berg (1967) (4). Much later, the *E. coli* gene encoding deacylase activity was identified (5). Disruption of this gene, called *dtd*, causes reduced growth of the bacterium on minimal medium containing either D-tyrosine, D-aspartate or D-tryptophan (2,5).

Further studies showed that, in a Δdtd context and in the presence of D-tyrosine, nearly half of the pool of cellular tRNA specific of L-tyrosine was esterified with this D-amino acid, thus explaining the impairment of growth (3).

The product of *E. coli dtd* (DTD1) is called D-aminoacyl-tRNA deacylase or D-Tyr-tRNA^{Tyr} deacylase. It has been studied in depth (3,6). The reaction catalyzed by DTD1 is highly specific for the D-isomer of the amino acid esterified to tRNA. The enzyme is active with different substrates such as D-Tyr-tRNA^{Tyr}, D-Asp-tRNA^{Asp} or D-Trp-tRNA^{Trp}. Under optimal *in vitro* conditions, the hydrolysis of D-Tyr-tRNA^{Tyr} proceeds at a maximal rate of 6 s^{-1} with a K_m value of $1\text{ }\mu\text{M}$ (5).

Homologs of the *E. coli dtd* gene are recognizable in the genomes of many bacteria and eukaryotes, not in those of archaea. Another type of D-Tyr-tRNA^{Tyr} deacylase (DTD2) has been discovered in archaea (7). This enzyme displays the fold of a bacterial peptidyl-tRNA hydrolase and carries two firmly bound zinc ions, crucial to the activity (7). Despite marked differences from its bacterial counterpart, the structural gene of the archaeal hydrolase (*dtd2*) can complement an *E. coli* Δdtd mutant for resistance to D-tyrosine. Homologs of *dtd2* are found in most archaea and in plants.

In *Arabidopsis thaliana*, the *dtd2* homolog, previously named *GEK1*, has been shown to be involved in ethanol resistance (8,9). Mutants in which *GEK1* expression is impaired no longer germinate on a culture medium containing 0.04% ethanol. Mutant seedlings are 10–100-fold more sensitive to ethanol than wild-type plants while overexpression of *GEK1* improves the tolerance to ethanol (9). The ethanol hypersensitivity of the *gek1* mutants is attributed to enhanced sensitivity to acetaldehyde, a metabolite of ethanol (9). Remarkably, the *gek1* mutants do not display any particular behavior in response to anoxic, heat shock or salt stresses (9).

The *GEK1* product (GEK1) displays ~26% strict identity with available archaeal D-Tyr-tRNA^{Tyr} deacylase sequences (7). Moreover, residues essential to the activity

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of archaeal deacylases are conserved in GEK1. This strongly suggests that GEK1 carries the activity of a D-aminoacyl-tRNA deacylase. To test this idea, we expressed the *GEK1* product in an *E. coli* context. The purified recombinant protein proves to be a very active D-aminoacyl-tRNA deacylase *in vitro*. We also establish that expression of GEK1 functionally complements an *E. coli* Δdtd mutant. Possible metabolic pathways linking the deacylase activity of GEK1 to exposure of plants to acetaldehyde are discussed.

MATERIALS AND METHODS

D-[methylene- ^3H]tyrosine (211 GBq/mmol) was custom-prepared by Amersham Biosciences. D-[2,3- ^3H]aspartic acid (1.33 TBq/mmol) was directly purchased at Amersham Biosciences. L-[^{14}C]tyrosine (16 GBq/mmol) was from NEN Life Science Products and unlabeled D-tyrosine was from Sigma.

Strains and plasmids used in this study are listed in Table 1.

Cloning of the *GEK1* gene in the pET3alpa and in the pET15blpa vectors

To introduce the coding sequence of *GEK1* into the expression vectors pET3alpa and pET15blpa (13), the plasmid harbored by clone S81170 (provided by the Arabidopsis Biological Resource Center, (14)) was used as template in PCR amplifications. In view of the insertion into pET3alpa, the following two primers were chosen for amplification: NdeI-*GEK1*-for (5'-GGGAATTCCA TATGGTAACACTAATCGTGGCCACCGCCGATCC AGCGTCGATCAACCCTGC-3') and NotI-*GEK1*-rev (5'-TTTTCTTTTTCGCGCCGCTCATGTGAAATCG TTTGGCTTCCCA-3'). For the insertion into pET15blpa, primers were: SW1 (5'-CTAGTCTAGACT AGTTTAAGGAGATATACATATGGTAACACTAA TCGTGG-3') and NotI-*GEK1*-rev. The two resulting amplified fragments were purified with the help of the Qiagen PCR purification Kit 50 and digested by either NdeI plus NotI or by XbaI plus NotI. Digestion products were inserted into plasmid pET3alpa or pET15blpa to give plasmids pET3alpa::*GEK1* and pET15blpa::*GEK1*, respectively. In each plasmid, the cloned gene was verified by DNA sequencing.

Preparation of crude extracts

Bacteria were grown at 37°C in 75 ml of 2xTY medium containing 100 µg/ml of ampicillin until the stationary phase of growth. Then, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at a final concentration of 1 mM and the culture was further incubated at room temperature for 5 h with agitation. After centrifugation at 9800 g for 15 min at 4°C, bacteria were resuspended in 5 mM potassium phosphate (pH 7.2) containing 160 µM zinc acetate and 10 mM 2-mercaptoethanol (PZM buffer). The volume of this buffer was adjusted to obtain an optical density (OD) at 650 nm of ~100. Cells were disrupted by sonication (3 min, 0°C) and debris were removed by centrifugation (10 min, 20 600 g, 4°C).

Table 1. Strains and plasmids

| | Description | Reference |
|-------------------------------------|---|------------|
| Strains | | |
| <i>E. coli</i> strains | | |
| XL1-Blue | <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F' proAB lacI^q lacZΔM15 Tn10)</i> | Stratagene |
| K37 | <i>galK rpsL</i> | (10) |
| K37Δ <i>recA</i> λDE3 | K37 Δ <i>recA</i> 938::cat (λDE3) | (7) |
| K37Δ <i>recA</i> Δ <i>tyrH</i> λDE3 | K37 Δ <i>recA</i> 938::cat Δ <i>dtd</i> ::kan (λDE3) | (7) |
| <i>S. cerevisiae</i> strains | | |
| DBY2057 | <i>MATa ura3-52</i> | (11) |
| DBY2057Δ <i>DTD1</i> | DBY2057 Δ <i>dtd1</i> ::kan | (12) |
| Plasmids | | |
| pET3alpa | Ap ^R | (13) |
| pET15blpa | Ap ^R | (13) |
| pET3alpa:: <i>GEK1</i> | Ap ^R <i>GEK1</i> derivative of pET3alpa | This work |
| pET15blpa:: <i>GEK1</i> | Ap ^R <i>GEK1</i> derivative of pET15blpa | This work |

Deacylase activity was measured in the supernatant. The total amount of proteins in the extract was determined by using the Bradford protein assay (Biorad), with bovine serum albumin (BSA) as the standard.

Preparation of substrates

E. coli tRNA^{Tyr} and tRNA^{Asp} were prepared as described previously (2,5,15). tRNA^{Tyr} was aminoacylated with D-[^3H]tyrosine (500 Ci/mol) or L-[^{14}C]tyrosine (434 Ci/mol) as described (2,5) with the following modifications. To synthesize D-Tyr-tRNA^{Tyr}, the reaction mixture (1 ml) contained 20 mM Tris-HCl (pH 7.8), 7 mM MgCl₂, 2 mM ATP, 3.5 µM D-[^3H]tyrosine, 3.5 µM tRNA^{Tyr} and 1.2 µM purified *E. coli* tyrosyl-tRNA synthetase. L-[^{14}C]Tyr-tRNA^{Tyr} was prepared with a similar procedure, with the exception that L-[^{14}C]tyrosine, tRNA^{Tyr} and tyrosyl-tRNA synthetase were added to the reaction mixture at final concentrations of 10, 7 and 0.5 µM, respectively. D-Asp-tRNA^{Asp} was synthesized in a reaction mixture containing 20 mM Tris-HCl (pH 7.8), 7 mM MgCl₂, 2 mM ATP, 0.1 mM EDTA, 60 µM D-[^3H]aspartic acid (500 Ci/mol), 5 µM tRNA^{Asp} and 6.5 µM purified *E. coli* aspartyl-tRNA synthetase.

Reaction conditions (10 min, 28°C) allowed nearly full esterification of tRNA^{Tyr} by D- or L-tyrosine, and 20% esterification of tRNA^{Asp} by D-aspartic acid. Nucleic acids were precipitated with the addition of 2.5 volumes of ethanol and 300 mM sodium acetate (pH 4.8), and recovered by centrifugation (30 min, 20 600 g, 4°C). The pellets were resuspended in 50 µl of 20 mM sodium acetate (pH 4.8) containing 100 mM KCl and 0.1 mM EDTA. Finally, the samples were chromatographed on a Trisacryl GF05 column (0.25 × 16 cm) equilibrated in the same buffer. Recovered D-aminoacyl tRNAs were stored at -20°C.

Diacetyl-L-[^{14}C]Lys-tRNA^{Lys} (310 Ci/mol) was prepared as described previously (15).

Measurement of deacylation rates

Unless otherwise stated, D-Tyr-tRNA^{Tyr} hydrolysis by GEK1 was followed under initial rate conditions for 5 min at 28°C in 100 µl assays containing 50 nM [³H]D-Tyr-tRNA^{Tyr}, 20 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 40 µM zinc acetate, 50 µg/ml BSA and 2.5 mM 2-mercaptoethanol. Prior to its addition to the assay, the sample containing enzyme activity was diluted in 20 mM Tris-HCl (pH 7.8) containing 160 µM zinc acetate, 200 µg/ml BSA and 10 mM 2-mercaptoethanol. The reaction was quenched by the addition of 340 µl ethanol, 14 µl sodium acetate 3 M (pH 4.8) and 20 µl carrier RNA from yeast at 4 mg/ml. Samples were centrifuged (20 min, 20 600 g, 4°C) and the radioactivity in the supernatant was measured by scintillation counting (5). Initial rates of L-[¹⁴C]Tyr-tRNA^{Tyr}, D-[³H]Asp-tRNA^{Asp} or diacetyl-L-[¹⁴C]-Lys-tRNA^{Lys} hydrolysis were measured under the same conditions.

To measure the catalytic constants of purified GEK1 in the reaction of D-Tyr-tRNA^{Tyr} hydrolysis, initial rates were followed as above in miniaturized (20 µl) assays containing increasing concentrations of the substrate (100–4000 nM) and GEK1 concentrations ranging from 3 to 6 pM. *K_m* and *k_{cat}* values were derived from iterative non-linear fits of the theoretical Michaelis equation to the experimental values using the Levenberg–Marquardt algorithm (16).

E. coli DTD1 deacylase activity was assayed at 28°C for 5 min in 20 mM Tris-HCl (pH 7.8) containing 5 mM MgCl₂, 0.025 mM EDTA, 50 µg/ml BSA and 2.5 mM 2-mercaptoethanol. Prior to its addition to the assay, the enzyme was diluted in 20 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 200 µg/ml BSA and 10 mM 2-mercaptoethanol.

Pyrococcus abyssi DTD2 deacylase activity was measured at 37°C for 5 min in 20 mM Tris-HCl (pH 7.8) containing 4 mM MgCl₂, 40 µM zinc acetate, 50 µg/ml BSA and 2.5 mM 2-mercaptoethanol. Prior to its addition to the assay, the enzyme was diluted in 20 mM Tris-HCl (pH 7.8) containing 160 µM zinc acetate, 200 µg/ml BSA and 10 mM 2-mercaptoethanol.

Purification of GEK1

E. coli strain K37ΔrecAΔtyrHΔDE3 was transformed by plasmid pET3alpa::GEK1. Transformed cells were grown at 37°C in 1 l of 2 × TY medium containing 100 µg/ml ampicillin. When the OD₆₅₀ of the culture reached 1.2, IPTG was added at a final concentration of 1 mM and growth was continued for 5 h at room temperature. Cells were harvested by centrifugation (15 min, 11 300 g, 4°C) and resuspended in PZM buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells (OD₆₅₀ = 50) were disrupted by sonication (10 min, 0°C) and debris removed by centrifugation (40 min, 7700 g, 4°C). Nucleic acids were precipitated by the addition of streptomycin (30 mg/ml) to the supernatant. Then, the sample was centrifuged for 20 min at 17 400 g (4°C). The resulting supernatant was brought to 70% ammonium sulfate saturation and centrifuged for 15 min at 17 400 g (4°C). The protein pellet was dissolved in 20 ml of PZM

buffer and dialyzed against 3 l of the same buffer. The resulting solution was applied on a column of Q-Sepharose Fast Flow (3.2 × 11.2 cm, Pharmacia) equilibrated in PZM buffer. Elution was performed at a flow rate of 1.3 ml/min with a 1.5 l linear NaCl gradient (0–0.5 M NaCl in PZM buffer). Enzyme activity was recovered between 208 and 272 min of the gradient. Active fractions were pooled and immediately applied on a hydroxylapatite column (1.1 × 10 cm, Biorad) equilibrated in PZM buffer. This column was eluted at a flow of 0.2 ml/min with 100 ml of a linear potassium phosphate buffer gradient (5–500 mM potassium phosphate, pH 7.2, in PZM buffer). Enzyme activity recovered between 165 and 260 min of the gradient was pooled. According to SDS-PAGE analysis, the purified protein was at least 95% homogeneous. The protein was then concentrated by ammonium sulfate precipitation (70% saturation). After centrifugation at 17 400 g for 30 min (4°C), the pellet was dialyzed twice, first against PZM buffer to eliminate ammonium sulfate and second against PZM buffer containing 60% glycerol. The purification procedure yielded 6.6 mg of protein from 1.7 g (wet weight) cells. Storage was at –20°C.

Concentrations of GEK1 were calculated using a molecular weight of 34 726 Da and an extinction coefficient of 1.737 at 280 nm.

Zinc concentration measurements

Zinc content of GEK1 was estimated from mass spectrometry experiments. An aliquot of GEK1 in 70% ammonium sulfate was dialyzed overnight against 10 mM ammonium acetate (pH 7.5) containing 10 µM zinc acetate. Then, zinc measurements were performed on a Bruker APEX III FT-ICR mass spectrometer equipped with a 7.0 T actively shielded magnet and an unmodified Apollo electrospray source, as previously described (7). The mass was determined under native or denaturing (50% acetonitrile and 0.2% formic acid) conditions.

Zinc in GEK1 was also assessed by flame atomic absorption spectroscopy. In this case, prior to zinc analysis, GEK1 was submitted to a size-exclusion chromatography on a TSK SWXL 3000 column equilibrated in a zinc-depleted buffer (20 mM Tris-HCl, pH 7.8, 300 mM KCl), as described previously in the case of an archaeal DTD2 protein (7). The eluted protein was followed through its absorbance at 280 nm and through measurement of its deacylase activity. Active fractions were submitted to zinc analysis at 213.9 nm, in the peak height mode, with 90 µl injections, using a Varian AA220 spectrophotometer equipped with an air–acetylene burner. Various zinc solutions (0–40 µM) in the above buffer were used as standards.

Measurement of D-tyrosine or D-aspartic acid toxicity

E. coli cells were grown overnight at 37°C in M9-glucose minimal medium containing 100 µg/ml of ampicillin, plus either 2.4 mM D-tyrosine or 11 mM D-aspartic acid. Control experiments without D-amino acid were performed in parallel. Then, bacteria were diluted in the same media containing or not a D-amino acid, to a final OD₆₅₀

of 0.05 and left to grow at 37°C. The OD₆₅₀ of the cultures were measured every 90 min, during 540 min, to calculate generation times.

Effect of ethanol and acetaldehyde on the growth of various *E. coli* and *Saccharomyces cerevisiae* strains

E. coli strains K37ΔrecAλDE3 and K37ΔrecAΔtyrHλDE3 were plated on M9 minimal medium supplemented with 0.2% glucose and 0–16% ethanol or 0–12% acetaldehyde. Then, cells were left to grow for a week at 37°C. Possible effects of ethanol or acetaldehyde on the growth at 30°C of *S. cerevisiae* strains DBY2057 and DBY2057ΔDTD1 were searched for in the same manner, except that a yeast nitrogen base supplemented with 50 μg/ml uracil and 2% glucose was used as the growth medium.

RESULTS

Expression of GEK1 in *E. coli*

To express GEK1 in *E. coli*, the coding sequence of the *GEK1* gene was inserted into either the pET3alpa or the pET15blpa expression vector, under control of the T7 promoter. After introduction of the resulting plasmids in the Δ*tdt* *E. coli* strain K37ΔrecAΔtyrHλDE3, GEK1 expression was induced by addition of IPTG. Cells grown in a rich culture medium were harvested after 5 h of induction, and deacylase activity in crude cell extracts was measured. Extracts from strains K37ΔrecAλDE3 (*tdt*⁺) and K37ΔrecAΔtyrHλDE3 each containing either plasmid pET3alpa or plasmid pET15blpa were also examined (Table 2). In the presence of plasmids pET3alpa::GEK1 or pET15blpa::GEK1, activities in cell extracts against D-Tyr-tRNA^{Tyr} as substrate were increased by a factor of at least 40 000, compared to the activity of the crude extract from the Δ*tdt* mutant, or by a factor of 4000, compared to the activity of the crude extract from the strain containing the endogeneous *E. coli* deacylase.

These results establish that, in an *E. coli* context, a protein with D-Tyr-tRNA hydrolase activity can be produced from the open reading frame of *GEK1*.

Activity of purified GEK1 protein

To precisely measure the deacylase activity of GEK1, this protein was purified to homogeneity. Under initial rate conditions (28°C, 20 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 50 nM D-[³H]Tyr-tRNA^{Tyr}, 40 μM zinc acetate, 50 μg/ml BSA, 2.5 mM 2-mercaptoethanol and 3–6 pM of enzyme), the activity of the purified GEK1 protein was 12.7 s⁻¹.

Previous experiments showed that ionic strength stimulated the activities of *E. coli* and *P. abyssi* D-Tyr-tRNA^{Tyr} deacylases, probably through the folding of the tRNA 3D structure (5,7). In order to determine the effect of ionic strength on the activity of GEK1, initial rates of D-Tyr-tRNA^{Tyr} hydrolysis were measured in the presence of various MgCl₂ and KCl concentrations. Addition of one or the other of these salts strongly stimulated the deacylase activity (Table 3). At optimal concentrations of MgCl₂ or KCl (5 and 50 mM, respectively), the

Table 2. Deacylase activity in crude extracts

| Strain | Deacylase activity (U/mg) |
|-------------------------------------|---------------------------|
| K37ΔrecAλDE3 + pET3alpa | 0.9 |
| K37ΔrecAΔtyrHλDE3 + pET3alpa | <0.1 |
| K37ΔrecAΔtyrHλDE3 + pET3alpa::GEK1 | 7700 |
| K37ΔrecAλDE3 + pET15blpa | 1.0 |
| K37ΔrecAΔtyrHλDE3 + pET15blpa | <0.1 |
| K37ΔrecAΔtyrHλDE3 + pET15blpa::GEK1 | 4000 |

Cells were grown overnight at 37°C in 2xTY medium with 100 μg/ml ampicillin. Then, IPTG was added at a final concentration of 1 mM, and growth was continued for 5 h at room temperature. Specific deacylase activities were measured in crude extracts obtained by sonication. Final total protein concentration in the extracts was 10–20 mg/ml. Rates of hydrolysis of D-[³H]Tyr-tRNA^{Tyr} in the presence of 20 mM Tris-HCl (pH 7.8), 100 nM D-[³H]Tyr-tRNA^{Tyr}, 5 mM MgCl₂, 40 μM zinc acetate, 50 μg/ml BSA and 2.5 mM 2-mercaptoethanol were measured as described in Materials and Methods. One unit corresponds to the enzyme activity capable of hydrolyzing 1 pmol of D-[³H]Tyr-tRNA^{Tyr} per min under the above assay conditions.

Table 3. Activity of GEK1 under various assay conditions

| Added component | Initial rate (s ⁻¹) |
|---|---------------------------------|
| None | 1.3 |
| 3 mM MgCl ₂ | 12.1 |
| 5 mM MgCl ₂ | 12.7 |
| 7 mM MgCl ₂ | 11.3 |
| 10 mM MgCl ₂ | 8.3 |
| 15 mM MgCl ₂ | 3.7 |
| 20 mM MgCl ₂ | 3.4 |
| 20 mM KCl | 5.6 |
| 50 mM KCl | 10.4 |
| 100 mM KCl | 9.1 |
| 200 mM KCl | 2.7 |
| 400 mM KCl | 0.3 |
| 5 mM MgCl ₂ + 20 mM KCl | 9.6 |
| 5 mM MgCl ₂ + 50 mM KCl | 6.1 |
| 5 mM MgCl ₂ + 100 mM KCl | 3.1 |
| 5 mM MgCl ₂ + 200 mM KCl | 1.2 |
| 5 mM MgCl ₂ + 400 mM KCl | 0.1 |
| 40 μM zinc acetate | 1.7 |
| 250 μM EDTA | 1.3 |
| 5 mM MgCl ₂ + 40 μM zinc acetate | 11.9 |
| 5 mM MgCl ₂ + 250 μM EDTA | 13.7 |
| 5 mM MgCl ₂ + 40 μM zinc acetate + 250 μM EDTA | 11.9 |
| 50 mM KCl + 40 μM zinc acetate | 9.5 |
| 50 mM KCl + 250 μM EDTA | 11.7 |
| 50 mM KCl + 40 μM zinc acetate + 250 μM EDTA | 10.1 |

Initial rates of hydrolysis of D-[³H]Tyr-tRNA^{Tyr} catalyzed by purified GEK1 were measured at 28°C for 5 min in the presence of 20 mM Tris-HCl (pH 7.8), 50 nM D-[³H]Tyr-tRNA^{Tyr} and the indicated components. Prior to the assay, GEK1 was diluted in 20 mM Tris-HCl buffer (pH 7.8) containing 200 μg/ml BSA and 10 mM 2-mercaptoethanol. Shown values are within ±15%.

deacylase activity was increased by factors of ~10 and ~8, respectively, with respect to assay conditions without any added salt. The simultaneous addition of MgCl₂ and KCl did not increase further the reaction rate. On the contrary, addition of KCl in the presence of the optimal MgCl₂ concentration (5 mM) led to a diminution of the deacylase activity. Such experiments suggest that ionic strength improves the rate of the reaction through

formation of a native tRNA structure. At excessive ionic strength, the enzyme activity becomes impaired probably through inhibition of the binding of the substrate.

Because the archaeal deacylase carries strongly bound zinc ions, we examined whether GEK1 needed these metal ions to display full activity. The initial rate of D-Tyr-tRNA^{Tyr} hydrolysis by the plant hydrolase was not sensitive to the presence in a 5-min assay of either 40- μ M zinc acetate or 250- μ M EDTA, an efficient chelator of zinc ions. In another set of experiments, GEK1 was left to incubate in the presence of 1 mM EDTA prior to the assay (Figure 1). At various times, aliquots were withdrawn and assayed (5 min) for deacylase activity. Measured activity progressively decreased, with a half-life time of ~ 3 h. After 24 h, almost 100% of the initial activity was lost. However, if the inactivated enzyme was assayed in the presence of 40 μ M zinc acetate, nearly full activity could be recovered. If GEK1 was incubated with 160 μ M zinc acetate instead of 1 mM EDTA, enzyme activity remained constant for at least 24 h. This behavior markedly resembles that of an archaeal DTD2 deacylase (7). It strongly suggests that zinc ions are essential to the activity of the protein.

Strong association of zinc to GEK1

Archaeal DTD2 enzymes appear to contain two zinc ions. Thus, we examined whether GEK1 also carried zinc. For this purpose, a sample of GEK1 was dialyzed overnight against 10 mM ammonium acetate buffer (pH 7.5) containing 10 μ M zinc acetate and submitted to mass spectrometry under denaturing or non-denaturing conditions. The difference between the denatured mass (34597 Da) and the native mass (34726 Da) exactly corresponded to the mass of two zinc atoms (65 Da).

The strong association of zinc to GEK1 was confirmed by flame atomic absorption spectroscopy. A sample of GEK1 was subjected to gel filtration in 20 mM Tris-HCl (pH 7.8) buffer containing 300 mM KCl. We verified that the specific activity of GEK1 was not modified during this chromatographic step. Then, active collected fractions were analyzed for zinc content. Based on the theoretical A_{280} extinction coefficient of GEK1, a stoichiometry of 2.0 ± 0.2 mol of zinc per mol of GEK1 was found.

Altogether, the above results establish that GEK1 is tightly associated with two zinc ions.

Specificity and catalytic constants of GEK1

Rates of D- and L-Tyr-tRNA^{Tyr} hydrolysis were compared at the same substrate concentration (100 nM) in assay conditions including 20 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 40 μ M zinc acetate, 50 μ g/ml BSA, 2.5 mM 2-mercaptoethanol, and 3 pM–30 nM of GEK1 (Table 4). The rate of L-Tyr-tRNA^{Tyr} deacylation was at least 25 000-fold lower than that of D-Tyr-tRNA^{Tyr} hydrolysis. Thus, GEK1 displays strict specificity towards the D-isomer of the amino acid. Diacetyl-L-Lys-tRNA^{Lys} also fully resisted the action of the deacylase. When assayed in the same conditions as above with 3–30 nM of enzyme, the specific activity of GEK1 towards this model substrate of peptidyl-tRNA hydrolases was

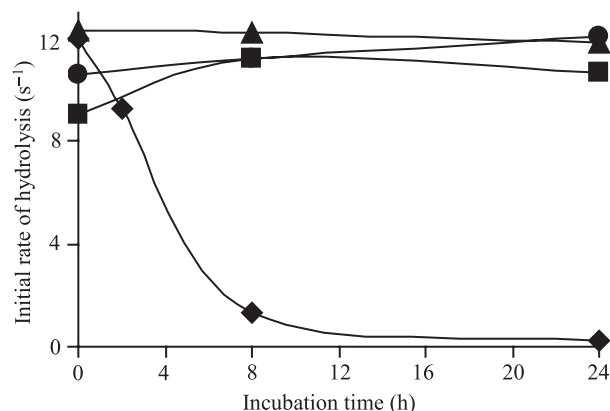


Figure 1. Incubation of GEK1 in the presence of EDTA or of zinc acetate: effect on the initial rate of D-Tyr-tRNA^{Tyr} hydrolysis. GEK1 (2 μ M) was incubated in 20 mM Tris-HCl buffer (pH 7.8) containing 200 μ g/ml BSA, 10 mM 2-mercaptoethanol and either 1 mM EDTA or 160 μ M zinc acetate. At the times indicated, an aliquot of GEK1 was withdrawn and assayed for 5 min in the presence of 20 mM Tris-HCl (pH 7.8), 50 nM D-[³H]Tyr-tRNA^{Tyr}, 5 mM MgCl₂ and either 250 μ M EDTA or 40 μ M zinc acetate. Filled square represents zinc acetate incubation followed by activity measurement in the presence of zinc acetate, filled triangle represents Zinc acetate incubation followed by activity measurement in the presence of EDTA, filled circle represents EDTA incubation followed by activity measurement in the presence of zinc acetate, filled diamond represents EDTA incubation followed by activity measurement in the presence of EDTA.

Table 4. Specificity of GEK1 towards the amino acid esterified to tRNA

| Substrate | Initial rate (s ⁻¹) |
|------------------------------------|---------------------------------|
| D-Tyr-tRNA ^{Tyr} | 25 |
| L-Tyr-tRNA ^{Tyr} | $<10^{-3}$ |
| Diacetyl-L-Lys-tRNA ^{Lys} | $<1.6 \times 10^{-4}$ |

Initial rates of hydrolysis of D-[³H]Tyr-tRNA^{Tyr}, L-[¹⁴C]Tyr-tRNA^{Tyr} or diacetyl-L-[¹⁴C]Lys-tRNA^{Lys} catalyzed by purified GEK1 (3–6 pM) were measured at 28°C for 5 min in the presence of 20 mM Tris-HCl (pH 7.8), 100 nM substrate, 5 mM MgCl₂, 40 μ M zinc acetate, 50 μ g/ml BSA and 2.5 mM 2-mercaptoethanol. Prior to the assay, GEK1 was diluted in 20 mM Tris-HCl (pH 7.8) containing 160 μ M zinc acetate and 200 μ g/ml BSA. Shown values are within $\pm 15\%$.

at least 150 000-fold lower than that towards D-Tyr-tRNA^{Tyr}.

Measurement of the rate of hydrolysis as a function of D-Tyr-tRNA^{Tyr} concentration (100–4000 nM) enabled us to deduce K_m and k_{cat} values of 1.1 ± 0.1 μ M and 300 ± 12 s⁻¹, respectively (data not shown). In the same conditions, the spontaneous chemical hydrolysis of D-Tyr-tRNA^{Tyr} occurred at a rate of 0.012 s⁻¹.

Therefore, *in vitro*, GEK1 displays a markedly high activity (28°C). Its k_{cat} value for hydrolysis is 50-fold higher than that of *E. coli* deacylase assayed under similar conditions (5). It is nearly 500-fold higher than that of *P. abyssi* deacylase as measured at 37°C (7). Possibly, such a high activity compensates for reduced *GEK1* expression

Table 5. Comparison of the substrate specificity of *A. thaliana*, *P. abyssi* and *E. coli* D-aminoacyl-tRNA deacylases

| | Deacylase activity (s^{-1}) | |
|--------------------------------------|---------------------------------|---------------------------|
| | D-Tyr-tRNA ^{Tyr} | D-Asp-tRNA ^{Asp} |
| <i>A. thaliana</i> GEK1 ^a | 12.7 ± 1.2 | 8.6 ± 2.4 |
| <i>P. abyssi</i> DTD2 ^b | 0.33 ± 0.05 | 0.12 ± 0.03 |
| <i>E. coli</i> DTD1 ^c | 0.25 ± 0.02 | 0.6 ± 0.05 |

Initial rates of hydrolysis were measured in the presence of 50 nM D-aminoacyl-tRNA.

^a28°C, 3–6 pM GEK1, 20 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 40 μM zinc acetate, 50 μg/ml BSA, 2.5 mM 2-mercaptoethanol. ^b37°C, 200–400 pM DTD2, 20 mM Tris-HCl, pH 7.8, 4 mM MgCl₂, 40 μM zinc acetate, 50 μg/ml BSA, 2.5 mM 2-mercaptoethanol. ^c28°C, 70–200 pM DTD1, 20 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 0.025 mM EDTA, 50 μg/ml BSA, 2.5 mM 2-mercaptoethanol.

in a plant cytoplasm. Expression of *GEK1* in various tissues of wild-type *Arabidopsis* was followed using northern blot analysis (9). The mRNA of *GEK1* was ubiquitously detected. However, its exact abundance was not evaluated.

To further examine the specificity of GEK1, we tested whether this protein was able to also use D-Asp-tRNA^{Asp} as substrate. The initial rate of hydrolysis of 50 nM D-Asp-tRNA^{Asp} was equal to 8.6 s⁻¹ (Table 5), a value very close to that obtained with 50 nM D-Tyr-tRNA^{Tyr} (12.7 s⁻¹). The specificity of GEK1 towards its substrate was compared to those of *E. coli* DTD1 and *P. abyssi* DTD2 under the same conditions of D-aminoacyl-tRNA concentration (50 nM). As shown in Table 5, these two proteins also hydrolyzed D-Asp-tRNA^{Asp} almost as efficiently as they hydrolyzed D-Tyr-tRNA^{Tyr}. We could therefore conclude that, similarly to a DTD1 enzyme (2), the DTD2 protein from archaea and the GEK1 protein from plants display broad specificity towards substrates carrying very different D-amino acid moieties.

Complementation of an *E. coli* Δdtd null mutant by GEK1 expression

To confirm the activity of the *GEK1* product *in vivo*, we studied the effect of GEK1 expression on the sensitivity of an *E. coli* Δdtd strain to D-tyrosine or D-aspartic acid. In minimal growth medium, the generation time of the strain was significantly increased in the presence of 2.4 mM D-tyrosine (2,3,5). However, our attempts to cultivate strain K37 Δ recA Δ tyrH Δ DE3 containing pET3alpa::GEK1 failed. It is likely that excess production of D-aminoacyl-tRNA deacylase activity from this plasmid impairs *E. coli* growth in minimal medium, as already reported (5). To alleviate this difficulty and obtain viable cells containing the *GEK1* product, we used strain K37 Δ recA Δ tyrH Δ DE3 carrying pET15blpa::GEK1. The pET15blpa plasmid differs from the pET3alpa one by the presence of *lacI*. The K37 Δ recA Δ tyrH Δ DE3 derivative harboring the pET15blpa::GEK1 plasmid was found to grow normally in minimal medium. Attenuation of *GEK1* transcription by the lactose repressor may explain this

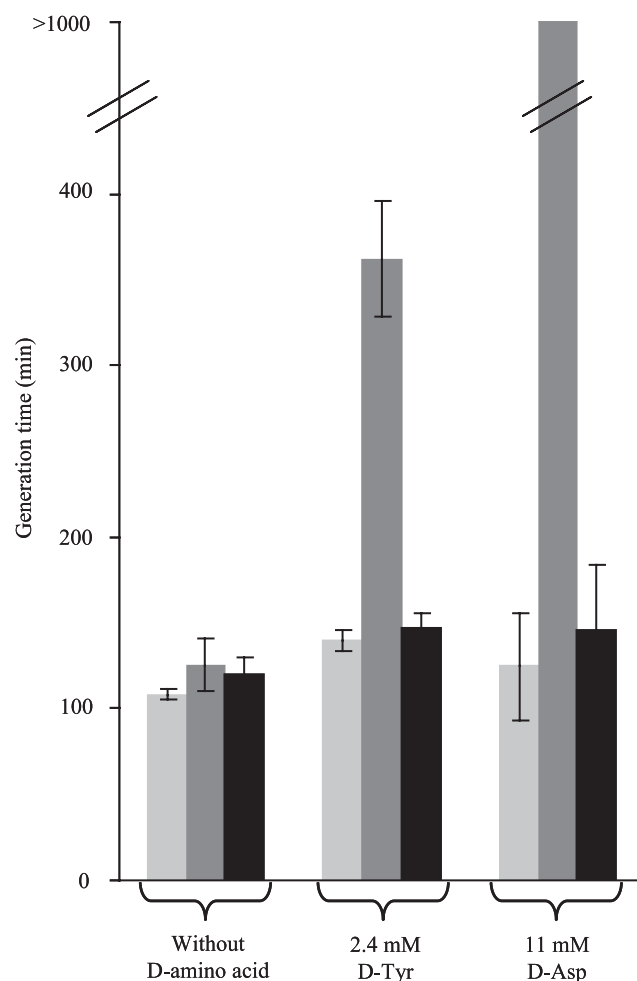


Figure 2. Generation time of various *E. coli* strains in the presence or absence of D-amino acids in the growth medium. Strains K37 Δ recA Δ DE3 (*dtd*⁺) or K37 Δ recA Δ tyrH Δ DE3 (Δdtd) cells containing pET15blpa or pET15blpa::GEK1 were grown at 37°C in M9-glucose minimal medium containing 100 μg/ml ampicillin and either 2.4 mM D-tyrosine or 11 mM D-aspartic acid. A control without D-amino acid was also performed. Cells were pre-grown overnight in the growth medium under study. For generation time measurements, inoculations were adjusted to an OD₆₅₀ of 0.05. Samples were withdrawn from the cultures every 90 min during 540 min, and generation times were deduced from OD₆₅₀ measurements. Shown experiments were performed using wild-type strain (K37 Δ recA Δ DE3) carrying pET15blpa (light bars), Δdtd mutant strain (K37 Δ recA Δ tyrH Δ DE3) carrying pET15blpa (middle dark bars) and Δdtd mutant strain (K37 Δ recA Δ tyrH Δ DE3) carrying pET15blpa::GEK1 (dark bars). Errors bars represent standard deviations calculated from two independent experiments. In the case of the strain K37 Δ recA Δ tyrH Δ DE3 carrying pET15blpa grown in the presence of 11 mM D-aspartic acid, the increase in the OD₆₅₀ value was less than 35% after 540 min. Therefore, only an approximate value of the generation time could be estimated.

behavior. Generation times of this strain in the absence or presence of D-amino acid (2.4 mM D-tyrosine or 11 mM D-aspartic acid) were compared with those of strains K37 Δ recA Δ DE3 and K37 Δ recA Δ tyrH Δ DE3 containing the control plasmid pET15blpa (Figure 2). In the absence of D-amino acid, the generation times of all three strains were identical (118 ± 7 min). As expected,

the generation time of the Δdtd mutant was longer in the presence of D-amino acid (2.3-fold in the presence of D-tyrosine and more than 9-fold in the presence of D-aspartic acid). Upon introduction of the plasmid producing GEK1, the Δdtd mutant exposed to D-tyrosine or D-aspartic acid recovered the generation time displayed in the absence of D-amino acid.

Thus, expression of *GEK1* in *trans* does rescue the sensitivity of a Δdtd mutant to D-tyrosine as well as to D-aspartic acid.

***E. coli* and *S. cerevisiae* strains lacking endogenous D-Tyr-tRNA^{Tyr} deacylase do not display any particular sensitivity to ethanol or to acetaldehyde**

Previous results have established a link between the *in vivo* function of GEK1 and sensitivity of a plant to ethanol or acetaldehyde (8,9). This link appears to be plant specific. Indeed, overexpression of *GEK1* in *E. coli* or yeast strains does not confer any particular phenotype in response to the addition of ethanol (9). However, since the latter experiments were performed in wild-type contexts, a dominant effect of the *E. coli* or yeast endogenous deacylases on ethanol tolerance cannot be excluded.

In the present study, to determine whether the endogenous deacylase function in *E. coli* or *S. cerevisiae* has any link with the ethanol response, we compared cells expressing or not their native *dtd/DTD1* gene. Wild-type *E. coli* strain and a derived Δdtd mutant (K37 Δ recA Δ DE3 and K37 Δ recA Δ tyrH Δ DE3, respectively) were grown in the presence of 0–16% ethanol or of 0–12% acetaldehyde in minimal medium at 37°C. Plates were surveyed for a week. We found that the growth rates of the two strains were identical whatever the added concentration of ethanol or acetaldehyde. Minimal inhibitory concentrations (MIC) were 12 and 2%, respectively.

Similar observations were made with *S. cerevisiae*. The growth rates of a *dtd1* mutant (DBY2057 Δ DTD1) and of its parental strain (DBY2057), at 30°C, in minimal medium containing various concentrations of ethanol or acetaldehyde, were indistinguishable. With both strains, MICs were 16% ethanol and 4% acetaldehyde.

Thus, we conclude that, in *E. coli* and yeast, D-Tyr-tRNA^{Tyr} deacylase activity does not sustain the metabolic link with ethanol that the GEK1 product does in *A. thaliana*.

DISCUSSION

The results of this study establish that the *A. thaliana* GEK1 protein behaves as a broad-specific, markedly active D-aminoacyl-tRNA deacylase. Therefore, the sequence homology between GEK1 and archaeal D-Tyr-tRNA^{Tyr} deacylases is accompanied by a functional homology. The resemblance between GEK1 and archaeal deacylases is reinforced by the occurrence in all proteins of zinc ions involved in enzymatic activity (7).

The genome of *A. thaliana* also contains a homolog of *dtd*, the *E. coli* deacylase gene. Therefore, *A. thaliana* is likely to express at least two enzyme species carrying D-Tyr-tRNA^{Tyr} deacylase activity. However, because cells

contain distinct compartments, the functions of these two enzymes may not be redundant. Indeed, a GEK1-green-fluorescent-protein fused protein was detected in the whole cell, including the nucleus (8), while, according to the computer program TargetP (17), the homolog of *E. coli* DTD1 should be localized to the chloroplast, with the help of a signal peptide of 42 amino acids. The endosymbiotic origin of chloroplasts and the resemblance between archaea and the cytosol of eukaryotes may account for segregation in two different compartments of two distinct enzymes sharing a same function.

GEK1 and the homolog of bacterial *dtd* co-exist in the genomes of all plants documented so far. This suggests that expression, in plant cells, of D-aminoacyl-tRNA deacylase activity is important to confer protection against D-amino acid toxicity. Sensitivity of plants to D-amino acids has already been documented. Growth and morphology are affected by exposure to D-amino acids (18). D-leucine causes 'leucinosis' in sunflowers, a disease characterized by chlorosis, smaller leaves and the appearance of necrotic regions on them (19). D-alanine, D-aspartic acid, D-glutamic acid and D-serine induce leaf shedding (20). Salt uptake by different plant tissues is inhibited by D-amino acids (18). Toxicity of D-amino acids depends on their side chain. For example, in *A. thaliana*, D-alanine and D-serine are very toxic whereas D-valine and D-isoleucine are not (21). In spite of these data, the D-amino acid metabolism of plants is still poorly understood. Most eukaryotic and prokaryotic organisms possess D-amino acid oxidase, a catabolic enzyme of D-amino acids (22). Such an enzyme has not been found in plants. Instead, plants possess a specific enzyme called L-aminocyclopropane-L-carboxylate *N*-malonyltransferase, which insures malonylation of D-amino acids, but not of L-amino acids (23–25). This modification is thought to be involved in the detoxification of D-amino acids.

Originally, GEK1 was identified because of its role in the ethanol stress response of *A. thaliana* (8,9). The deacylase function of GEK1 suggests a link between this ethanol stress response, D-amino acid toxicity and protein synthesis. Notably, several studies indicate that ethanol could affect protein synthesis in eukaryotes (26–29). On the basis of the present study, we have now to discover functional links between ethanol or acetaldehyde addition to a plant cell and the extent of D-amino acid transfer onto tRNA.

Several speculations can be made based on the property of acetaldehyde to react with NH₂ or SH groups (30–32). (i) A first possibility is an inhibition by acetaldehyde of the malonylating reaction mentioned above, with a resulting increase in the concentration of the D-amino acid pool in the cell. The malonyltransferase does not exist in *E. coli* or yeast. Thus, a participation of this enzyme in the behavior of the *gek1* mutant would account for a restriction to plants of the link between ethanol and D-amino acids. Possibly, inhibition of the *N*-malonyltransferase involves inactivation of its cofactor coenzyme A. In mouse brain extracts, coenzyme A has been found to be sensitive to relatively low concentrations of acetaldehyde (0.2 mM) (33). (ii) A second possibility is an upregulation of the production of D-amino acids as side-products of L-amino

acid synthetic pathways. For instance, chemical modification by acetaldehyde of an enzyme sustaining L-amino acid synthesis might change the ratio between the concentrations of D- and L-amino acids produced. (iii) A third hypothesis involves a change of the stereospecificity in the charging reaction of a tRNA, through chemical modification of the cognate aminoacyl-tRNA synthetase or of the tRNA itself. Improved transfer to the tRNA of the D-isomer of the amino acid would account for the toxicity of acetaldehyde towards a plant cell deprived of cytosolic D-aminoacyl-tRNA deacylase. (iv) A fourth possible hypothesis is a blocking reaction by acetaldehyde of the α -NH₂-groups of the amino acids esterified to tRNA. Such a modification is expected to confer improved resistance against spontaneous deacylation (5). Possibly, *in vivo*, such N-blocked L-aminoacyl-tRNA species are enzymatically recycled, but not the D-aminoacyl-tRNA ones (34). Thus, under this hypothesis, the cell would take advantage of the presence of a D-Tyr-tRNA^{Tyr} deacylase to ensure hydrolysis of D-aminoacyl-tRNAs before they are converted into long-lived, inactive species, by acetaldehyde attack.

Further studies must now be undertaken to identify which reaction(s) link D-aminoacyl-tRNAs to ethanol stress in *A. thaliana*. It is likely that such studies will shed new light on D-amino acid metabolism in plants.

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